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The association of thromboxane A_2 receptor with lipid rafts is a determinant for platelet functional responses

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ABSTRACT

We have investigated the presence of thromboxane A_2 (TXA_2) receptor associated with lipid rafts in human platelets and the regulation of platelet function in response to TXA_2 receptor agonists when lipid rafts are disrupted by cholesterol extraction. Platelet aggregation with TXA_2 analogs U46619 and IBOP was almost blunted in cholesterol-depleted platelets, as well as $\alpha_{IIb}\beta_3$ integrin activation and P-selectin exposure. Raft disruption also inhibited TXA_2-induced cytosolic calcium increase and nucleotide release, ruling out an implication of $P2Y_{12}$ receptor. An important proportion of TXA_2 receptor (40%) was colocalized at lipid rafts. The presence of the TXA_2 receptor associated with lipid rafts in platelets is important for functional platelet responses to TXA_2.

1. Introduction

The inhibition of platelet thromboxane A_2 (TXA_2) synthesis by aspirin constitutes the first front of antiplatelet treatment in patients with acute cardiovascular disease and, in secondary prevention, to reduce the risk of a new event among patients at high risk of occlusive vascular events [1]. TXA_2 is produced by activated platelets and acts by reinforcing platelet activation and by inducing the recruitment of new platelets to the growing thrombus. In addition, TXA_2 is a powerful vasoconstrictor. Actions of TXA_2 on platelets are mediated by the activation of TXA_2 receptor in the membrane of cells. The TXA_2 receptor belongs to the superfamily of seven transmembrane-domains receptors. In platelets, two isoforms of TXA_2 receptor (TP_α and TP_β) have been identified [2]. Both the TP_α and TP_β subtypes mediate the stimulation of phospholipase C and an increase in intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol. The formation of inositol 1,4,5-triphosphate induces an increase in the cytosolic concentration of Ca^{2+}, whereas the release of diacylglycerol activates PKC [2]. These actions of the TXA_2 receptors in platelets are mediated by the association of the receptor to G_q and G_{13} proteins [3].

The cell membrane microdomains called lipid rafts are sphingolipid and cholesterol-based structures consisting of very small domains (20–50 nm) of tightly packed lipids displaying lateral mobility [4]. Lipid raft formation should therefore be facilitated in membranes rich in cholesterol and sphingolipids that promote the formation of liquid-ordered domains in the presence of cholesterol [5]. Interestingly, there is a relatively high amount of sphingomyelin in human platelets plasma membrane as compared to other cell types, suggesting a lipid composition in favor of lipid raft formation in these cells [6]. Several previous studies have suggested that lipid rafts are highly dynamic platelet membrane structures involved in critical signaling mechanisms [4,7]. It has been reported that the presence of different proteins associated with lipid rafts, including membrane receptors (CD36, GPIb, GPVI, P2Y12), signal transduction partners (LAT, src, G proteins) and enzymes (PI3K, PLCγ_2) [6]. However, to the best of our knowledge, the presence of TXA_2 receptor in lipid rafts in platelets has not been previously described, although in other cell types the localization of TXA_2 receptors in lipid rafts have already been demonstrated [8,9].

The aim of this study was to explore the association of the TXA_2 receptor with lipid rafts in human platelets and the functional implications of this location for the platelet responses to TXA_2. Our results demonstrate, for the first time, the importance of lipid rafts for TXA_2-induced platelet responses, a process related with the presence of a fraction of TXA_2 receptors associated with lipid rafts.

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2. Methods

2.1. Antibodies

TXA_2 receptor (Cayman Chemicals, Ann Harbor, MI); CD36 (Santa Cruz Biotechnology, Dallas, TX); CD62-FITC, CD61-PE, CD42-phycoerythrin (PE) (Beckman Coulter, Barcelona, Spain); PAC-1-fluorescein isothiocyanate (FITC) (Becton Dickinson, Madrid, Spain).

2.2. Blood collection and platelet processing

Venous blood was obtained from healthy fasting donors, drug-free for at least 15 days, after informed consent, as approved by the institutional review board of the Hospital La Fe. Platelet isolation and washing were performed as described [10]. After washing, platelets were re-suspended in HBSS buffer (in mmol/L): 0.8 $MgSO_4$, 5.36 KCl, 0.441 KH_2PO_4, 137 NaCl, 0.34 Na_2HPO_4, 5.55 Glucose, 20 HEPES, pH 7.4.

2.3. Depletion of platelet cholesterol

Platelet rich plasma (PRP) was incubated (15 min., 37 °C) with 5 mM methyl-β-cyclodextrin (MβCD) (Sigma Aldrich, Madrid, Spain) [11]. Platelets were then isolated and re-suspended as described [10].

2.4. Platelet aggregation

Platelet aggregation was assessed by optical aggregometry in 300 μl of washed platelets (2×10^8 platelets/ml) in HBSS buffer supplemented with 1 mM $CaCl_2$ (final concentration) at 37 °C with constant stirring (1000 rpm) in a Chrono-Log 490–2D platelet aggregometer (Chrono-Log Corporation, Havertown, PA). The amplitude (percentage) of the platelet aggregation response was monitored up to 3 min. in reference to a buffer blank.

2.5. Dense granules release

Dense granules release was monitored by platelet nucleotide release as previously described [12]. Briefly, after 3 min. of platelet aggregation, samples were transferred to an eppendorf tube and centrifuged (1 min., 13 000×g), and the supernatant was collected. Perchloric acid (1:10 v/v, final concentration 0.3 N) was added to supernatants, kept in ice for 1 h, and the precipitated proteins were pelleted by centrifugation (8 min., 13 000×g, 4 °C). Supernatants were carefully neutralized with KOH, centrifuged (8 min., 13 000×g, 4 °C), and kept at −80 °C until further processing. Thawed samples were filtrated and injected in a Waters 600E HPLC system equipped with a C18 column (Teknokroma, Barcelona, Spain) and a UV detector Waters 486. Isocratic elution (0.4 ml/min., 30 °C) was performed with a mobile phase consisting of 200 mmol/L KH_2PO_4 adjusted to pH 6 with NH_4OH. Nucleotide elution was monitored at 254 nm (Lambda-Max Model 480, Waters–Waters Cromatografía SA, Barcelona, Spain). Concentration of nucleotides was calculated using a standard calibration curve of adenine nucleotides.

2.6. Flow cytometry

MβCD-free or MβCD-treated washed platelets (2×10^8 platelets/ml) in HBSS buffer plus 1 mmol/L $CaCl_2$ were incubated without stirring (10 min., 37 °C). Agonists were added, and incubation was continued for 5 min. without stirring. Duplicate 10-μL aliquots of stimulated platelets were transferred to polypropylene tubes that contained 100 μL HBSS buffer without calcium. To each sample, saturating concentrations of PAC-1-FITC or CD62-FITC plus a general platelet marker (CD42-PE for PAC-1 or CD61-PE for CD62 analysis) were added, kept undisturbed (30 min., 20 °C, dark), quench-diluted with 1 ml ice-cold HBSS, and maintained at 4 °C in the dark [10]. Platelets were gated based on size and CD42/CD61 fluorescence. Results are reported as percentages of platelets expressing PAC-1 or CD62 in a total of 5000 platelets per sample analyzed in an EPICS XL-MCL flow cytometer (Beckman Coulter, Madrid, Spain).

2.7. Measurement of cytosolic free Ca^{2+} concentration

PRP (treated or not with MβCD) was incubated with 1.5 μM FURA 2/AM (37 °C, 45 min.), washed and resuspended in HBSS buffer (75×10^5 platelet/ml) containing 1 mM calcium. Changes in FURA 2/AM fluorescence were continuously monitored after agonist addition by dual excitation fluorimetry at 340 and 380 nm, at 37 °C with stirring in an RF-1501 spectrofluorophotometer (Shimadzu, Duisburg, Germany), and the calcium concentration was calculated as described [13].

Isolation of lipid rafts fractions and identification of associated proteins was performed as previously described [14]. Washed platelets (4×10^8 platelets/ml) were lysed on ice with lysis buffer (final concentration (in mmol/L): 50 Tris–HCl pH 7.4, 100 NaCl, 5 EDTA, 50 NaF, 10 sodium pyrophosphate, 1 Na_3VO_4, 1% CHAPS supplemented with 1× protease inhibitor cocktail III (Merck Chemicals Ltd, Nottingham, UK). After complete rupture of platelets by aspirating repeatedly with a Hamilton syringe, lysates were mixed 1:1 with MNE buffer (MES (2-(N-morpholino) ethanesulfonic acid) 25 mmol/L pH 6.5, EDTA 5 mmol/L, NaCl 150 mmol/L) containing 80% sucrose. 1.5 ml of this mixture was laid on the bottom of an ultracentrifuge tube and sequencially overlaid carefully with 1.5 ml of 30% and 750 μl of 5% sucrose in MNE buffer. Samples were centrifuged (200 000×g, 18 h, 4 °C). Aliquots of 300 μl were carefully collected sequentially from the upper surface, mixed 1:1 with Laemmli sample buffer, and boiled 5 min. Equal volumes of sample were loaded on 4–12% polyacrylamide preformed gels (Life Technologies, Madrid, Spain), and the separated proteins were transferred to nitrocellulose membranes [10]. Immunodetection of CD36 and TXA_2 receptor were performed as previously described [10]. Images were scanned and quantified using the freely available public domain software ImageJ 1.45e (NIH, http:// rsbweb.nih.gov/ij/).

2.8. Statistical analysis

Significance was determined by Student's *t*-test. Results were expressed as mean ± S.E.M. of at least three different experiments.

3. Results

3.1. Regulation of thromboxane-induced platelet activation by the lipid rafts

To determine whether lipid rafts played a role in thromboxane-induced platelet activation, we investigated the effect of membrane cholesterol depletion on platelet aggregation, P-selectin exposure, and $\alpha_{IIb}\beta_3$ activation. We used two stable, structurally different analogs of thromboxane: U46619 (1 μM) and IBOP (10 nM) (Cayman Chemicals, Ann Arbor, MI). After cholesterol depletion with MβCD, U46119- and IBOP-induced aggregation were significantly inhibited (Fig. 1A). The different patterns of inhibition could be attributed to the different concentrations of the two thromboxane analogs. Interestingly, platelet aggregation induced

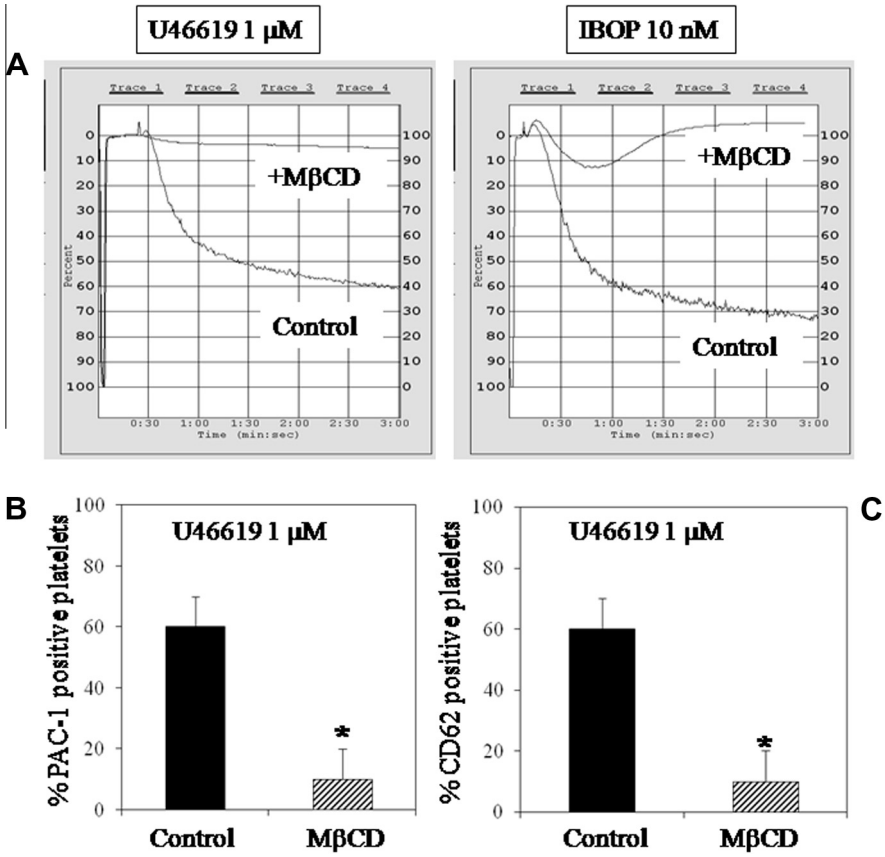

Fig. 1. Regulation of platelet responses to thromboxane by lipid raft disruption. PRP was treated with 5 mM MβCD (30 min, 37 °C) or solvent and platelets washed as described in Section 2. In (A), MβCD-treated or control platelets (2×10^8 platelets/mL) were stimulated with 1 μM U46619 or 10 nM IBOP and aggregation recorded up to 3 min. In (B) and (C), control or MβCD-treated platelets (2×10^7 platelets/mL) were stimulated with U46619 (1 μM) and incubated for 5 min. without stirring. Duplicate aliquots of stimulated platelets were diluted and incubated with PAC-1-FITC or CD62-FITC plus a general marker of platelets (CD42-PE for PAC-1 or CD61-PE for CD62), and flow cytometric determinations were performed as described in Methods. Results are media ± S.E.M. of percentage of platelets that bind CD62 or PAC-1 of 5 different experiments; $^*P < 0.05$. Disruption of lipid rafts inhibited platelet aggregation, $\alpha_{IIb}\beta_3$ activation and P-selectin exposure in response to U46619.

with high concentration of agonists (1 U/ml thrombin, 16 μg/ml collagen) was not affected by cholesterol depletion (results not shown) suggesting that platelet are functional after MβCD treatment.

As platelet aggregation is dependent on the activation of $\alpha_{IIb}\beta_3$, we used flow cytometry to assay the activation process of this integrin with the PAC-1 antibody, which specifically binds the activated conformation of $\alpha_{IIb}\beta_3$ [15] in control platelets and in platelets treated with MβCD. Fig. 1B shows that cholesterol depletion strongly reduced the U46619-induced activation of the $\alpha_{IIb}\beta_3$ integrin.

We next examined the role of cholesterol depletion on the release of α-granules by evaluating the exposure of P-selectin in the surface of platelets by flow cytometry. Results in Fig. 1C shows that exposure of P-selectin was significantly inhibited in cholesterol-depleted platelets.

3.2. $P2Y_{12}$-independent platelet responses to thromboxane are regulated by lipid rafts

It has been previously demonstrated that some platelet responses to thromboxane stimulation are at least partially dependent on the action of the released ADP upon its receptor $P2Y_{12}$ [16,17]. In addition, cholesterol depletion has been shown to reduce ADP-induced platelet aggregation through $P2Y_{12}$ receptor due to the presence of $P2Y_{12}$ [18] and Gi [17] associated with the lipid rafts. In accordance with this, Fig. 2A shows that blockade of $P2Y_{12}$ receptor with 2MeSAMP (10 μM) strongly inhibited U46619-induced washed platelet aggregation [19,20]. In contrast, other platelet responses to U46619 stimulation such as dense granule release (Fig. 2B) and increase in cytosolic calcium (Fig. 2C) are not significantly affected by the blockade of the $P2Y_{12}$ receptor in agreement with other authors [21]. Therefore, we concluded that thromboxane receptor responses are independent of $P2Y_{12}$. Then, we explored the effect of lipid rafts disruption on these platelets responses to U46619 that are independent of ADP release and $P2Y_{12}$ receptor activation (Fig. 2B and C). We found that MβCD-treatment significantly reduced platelet dense granule release evaluated by measurement of the release of ATP (Fig. 2D) and cytosolic calcium increase in FURA 2AM loaded platelets in response to U46619 (Fig. 2E). All together, the results in Figs. 1 and 2 suggested that the association of the thromboxane receptor with lipid rafts is important for the platelet responses to the eicosanoid.

3.3. The thromboxane receptor is partially associated with lipid rafts

To address the association of the thromboxane receptor with lipid rafts, lysed platelets were fractionated by sucrose density centrifugation in order to isolate the low density lipid raft membranes. The fractions corresponding to lipid rafts were identified using Western blotting by the presence of CD36, which is a generally accepted marker of platelet lipid rafts [22]. Western blotting of the same fractions showed that the thromboxane receptor was

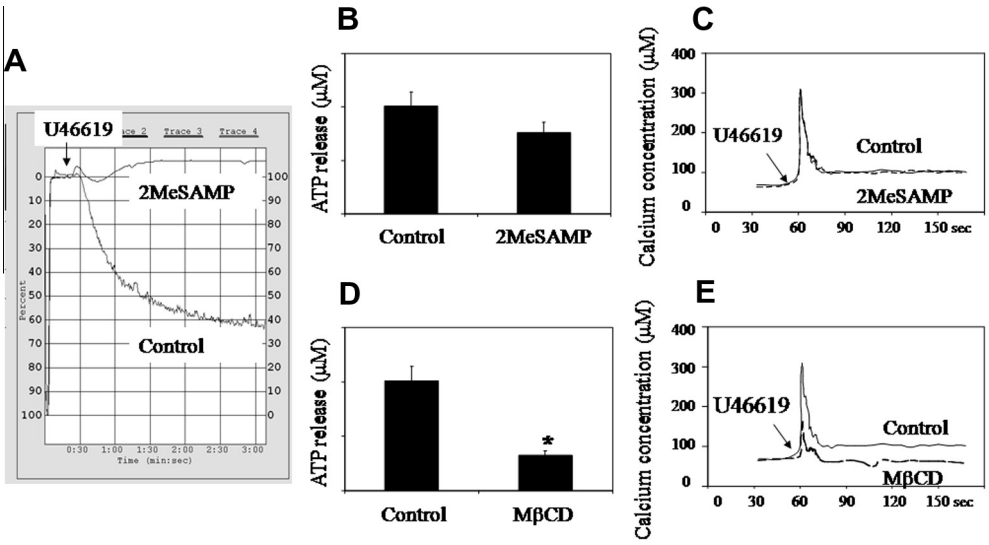

Fig. 2. Relevance of lipid rafts for thromboxane-induced platelet responses independent of the P2Y12 receptor. In (A), washed platelets were incubated (1 min, 37 °C) with 10 μM 2MeSAMP to block the $P2Y_{12}$ receptor or solvent and then stimulated with 1 μM U46619 and aggregation registered for 3 min. In (B) and (D), platelets incubated with 2MeSAMP (B) or MβCD (D) were stimulated with U46619 (1 μM) for 3 min., centrifuged, and ATP release determined by HPLC as described in Section 2. In (C) and (E), FURA 2AM-loaded platelets were incubated with 2MeSAMP (C) or MβCD (E) and stimulated with U46619 1 μM. Fluorescence was registered for 3 min. and transformed to calcium concentration as described in Section 2. Data in A are representative of 5 different analyses; data in B through D are media ± SEM of 3 different experiments; $^{*}P < 0.05$ MβCD-treated vs. control. Blockade of $P2Y_{12}$ receptor with 2MeSAMP strongly reduced U46619-induced platelet aggregation but did not affect dense granule release nor calcium increase, which are, therefore, $P2Y_{12}$-receptor independent responses. In contrast, disruption of lipid rafts with MβCD significantly inhibited these U46619-induced platelet responses independent of ADP.

partially localized in the lipid rafts (Fig. 3A). However, the thromboxane receptor was also present in other fractions not corresponding to lipid rafts. Densitometry of the fractions revealed that 39.5% of the thromboxane receptor was associated with lipid rafts (Fig. 3B; fractions 2–4). Furthermore, cholesterol depletion and lipid rafts disruption caused a redistribution of CD36 and thromboxane receptor to the heavier gradient fractions (Fig. 3C and D).

4. Discussion

The results in the present study show that the association of the thromboxane receptor with lipid rafts is important for platelet responses to thromboxane A_2. This is demonstrated by the observed inhibition of several platelet responses including platelet aggregation, dense and α-granule release, $\alpha_{IIb}\beta_3$ activation and the increase in cytosolic calcium in response to thromboxane analogs in cholesterol depleted platelets by MβCD treatment. In addition, our results demonstrate for the first time a partial association of the thromboxane receptor with lipid rafts in platelets.

The importance of thromboxane-induced activation of human platelets is underscored by the clinical benefit of the blockade of thromboxane synthesis with aspirin in patients with cardiovascular disease [1]. However, a variable percentage of aspirin-treated patients are not fully protected with aspirin, an effect probably related to an incomplete inhibition of thromboxane synthesis [13,19]. In addition, residual thromboxane synthesis [23] could cooperate with epinephrine to induce platelet aggregation [19], thus reducing the effectiveness of aspirin treatment. These results suggest that a better knowledge of the signal transduction

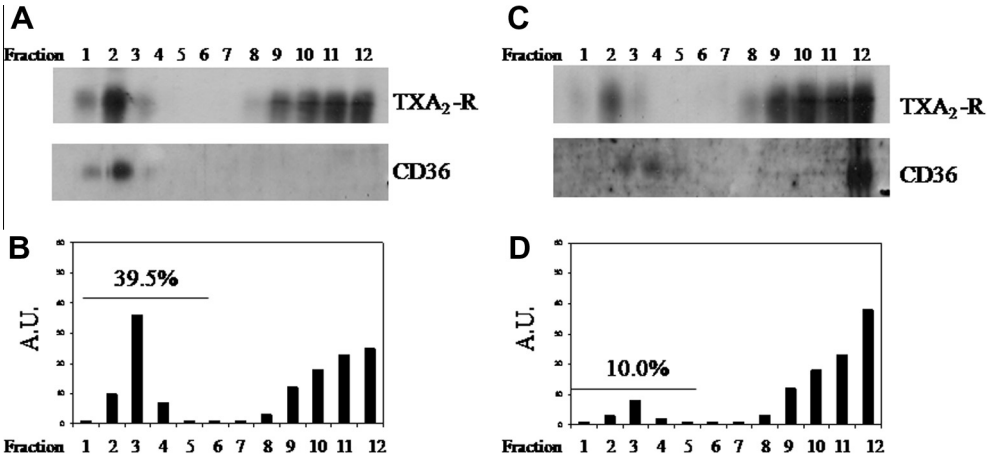

Fig. 3. Association of TXA_2 receptor with lipid rafts. In (A) and (B), control or MβCD-treated resting platelets were lysated, ultracentrifuged in a gradient of sucrose, and fractions separated and numbered from the upper layer (1–12). In (C) and (D), fractions corresponding to lipid rafts were identified by the presence of CD36. The presence of the TXA_2 receptor was determined by immunodetection. Images were quantified for the TXA_2 receptor and results expressed as arbitrary units (AU). Images are representatives of three different experiments. 39.5% of TXA_2 receptor was associated with lipid rafts.

mechanisms implicated in thromboxane signaling could provide new targets to improve aspirin therapy. Signaling through thromboxane receptor implies the participation of G_q and G_{13} but not of Gi, and activation of PLCγ [24]. Concerning platelet aggregation, some authors have suggested that full aggregation of platelets in response to thromboxane requires the participation of Gi activated through the action of released ADP upon its $P2Y_{12}$ receptor [16,20,21], a result that is confirmed in our study (Fig. 2A). In this respect, the presence of both $P2Y_{12}$ receptor [18] and Gi protein [17] associated with lipids rafts in human platelets has been previously described, as well as the importance of this location for ADP-induced activation of platelets [17]. However, as demonstrated in the present study other aspects of platelet responses to thromboxane such as dense granule release and increase in cytosolic calcium are independent of ADP release or $P2Y_{12}$ receptor activation (Fig. 2B and C), in agreement with other authors [16,20,21], and those ADP-independent responses are inhibited by lipid raft disruption (Fig. 2D and E). In the present study, we present evidence of the importance of the association of the thromboxane receptor with lipid rafts: (1) Disruption of lipid rafts with MβCD reduced platelet aggregation induced with U46619 and IBOP, two structurally different analogs of thromboxane (Fig. 1A); (2) MβCD treatment also reduced $\alpha_{IIb}\beta_3$ activation and P-selectin exposure (Fig. 1B); (3) cytoplasmic calcium increases and dense granule secretion, which are platelets responses to thromboxane independent of $P2Y_{12}$ signaling (Fig. 2B and C), were also significantly inhibited by cholesterol depletion (Figs. 2D and E); and (4) The thromboxane receptor appears associated, at least partially, with lipid rafts (Fig. 3). These results demonstrated, for the first time, that the association of the thromboxane receptor with lipid rafts is relevant for the agonistic effect of thromboxane in human platelets.

The presence of TXA_2 receptor associated with lipid rafts has been demonstrated in other cell types [8,9]. Our results demonstrated that in resting platelets only a percentage (39.5%) of thromboxane receptor is associated with lipid rafts, while a greater proportion is allocated outside of these structures. However, the presence of the thromboxane receptor associated with lipid rafts seems to be a key factor of its action. An interesting concern is the role of the main pool of TXA_2 receptor not associated with lipid rafts in resting platelets. In preliminary results in our laboratory we have observed that after platelet activation with different agonists, the proportion of TXA_2 receptor associated with lipid rafts increased dramatically (results not shown), an effect clearly reduced in MβCD treated platelets. We could speculate that the incorporation of TXA_2 receptor to lipid rafts after platelet activation could play an important role in platelet responses to TXA_2. The existence of two splice variants of thromboxane A_2 receptors, TPα and TPβ has been reported in humans, although the dominant form expressed in human platelets seems to be TP_α [25]. We have not performed experiments to detect the specific isoform of TXA_2 receptor that is incorporated into lipid rafts. In a recent report by Ibrahim et al. [9] described that activation of prostacyclin receptors induced polymerization with thromboxane receptors and redistribution to rafts in COS-7 cells, in a process related with cAMP levels. Interestingly, we have previously described that in patients with acute coronary syndrome treated with aspirin, activation of epinephrine receptors increased the platelet response to arachidonic acid [19]. These results suggest that epinephrine could also promote dimerization of TXA_2 receptors, although this point requires more investigations.

The presence of the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$ associated with the lipid rafts is at the present a controversial issue [22,26]. Our results show that disruption of lipid rafts strongly inhibited U46119-induced $\alpha_{IIb}\beta_3$ activation (Fig. 1B). More investigations are required to define the role of the possible association of $\alpha_{IIb}\beta_3$ with lipid rafts in thromboxane-stimulated platelets.

In conclusion, our results shown that association of a percentage of the thromboxane receptor with lipid rafts is a key factor for platelet responses to thromboxane.

Disclosure

None.

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